



## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

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<b>(21) International Application Number:</b> PCT/US99/23035  <b>(22) International Filing Date:</b> 4 October 1999 (04.10.99)  <b>(30) Priority Data:</b> 60/103,075                      5 October 1998 (05.10.98)                      US  <b>(71) Applicant (for all designated States except US):</b> MOSAIC TECHNOLOGIES [US/US]; 1106 Commonwealth Avenue, Boston, MA 02215 (US).  <b>(72) Inventors; and</b> <b>(75) Inventors/Applicants (for US only):</b> ABRAMS, Ezra, S. [US/US]; 4 Colbert Road, West Newton, MA 02465 (US). HAMMOND, Philip, W. [US/US]; 6 Hedgeway, Ayer, MA 01432 (US).  <b>(74) Agents:</b> HOGLE, Doreen, M. et al.; Hamilton, Brook, Smith & Reynolds, P.C., Two Militia Drive, Lexington, MA 02421 (US).		<b>(81) Designated States:</b> AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CR, CU, CZ, DE, DK, DM, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).  <b>Published</b> <i>With international search report.</i> <i>Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i>
<b>(54) Title:</b> REVERSE DISPLACEMENT ASSAY FOR DETECTION OF NUCLEIC ACID SEQUENCES  <div data-bbox="316 1180 1240 1478" data-label="Diagram"> <p>The diagram illustrates a four-step process for detecting a target nucleic acid sequence using a probe-tether complex.   <b>Target (1)</b> is represented by a wavy line.   <b>Tether-signal complex (2)</b> consists of a horizontal line (tether) with a small circle (signal) attached to it.   <b>Association:</b> An arrow points from the initial state to the second state, where the target (1) has hybridized with the first probe (a small circle) of the tether-signal complex.   <b>Branch Migration:</b> An arrow points to the third state, where the second probe (another small circle) of the tether-signal complex has hybridized with the target (1), displacing the first probe.   <b>Final State:</b> An arrow points to the fourth state, showing the <b>Tether:target Duplex (3)</b> (the horizontal line with the second probe) and the <b>Displaced Signal (4)</b> (the first probe). A plus sign (+) is shown between the duplex and the displaced signal.</p> </div>		
<b>(57) Abstract</b>  <p>The present invention pertains to a method and compositions for detecting the presence of a target nucleic acid in a test sample in which the sample to be tested for the presence of the target nucleic acid is introduced into a solution containing the probe-tether complex under conditions suitable for hybridization to occur between the first probe nucleic acid sequence and the target nucleic acid sequence so that the second tether nucleic acid sequence is displaced from the probe-tether complex, and a new complex is formed by the hybridization of the first probe nucleic acid sequence and the target nucleic acid sequence.</p>		

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## REVERSE DISPLACEMENT ASSAY FOR DETECTION OF NUCLEIC ACID SEQUENCES

### RELATED APPLICATIONS

This application claims the benefit of United States Provisional Application  
5 entitled "Reverse Displacement Assay for Nucleic Acid Targets", filed on October  
5, 1998 and assigned Serial Number 60/103,075. The teachings contained within  
that application, including all text, figures and references cited therein, are hereby  
incorporated by reference in their entirety.

### BACKGROUND OF THE INVENTION

10 The principle of hybridization is the property upon which most practical  
methods of detecting nucleic acid sequences are based. In general, methods for  
detecting the presence of particular target nucleic acid sequences involve employing  
a complementary sequence termed a probe, generally detectably labeled, and  
incubating this labeled probe sequence with the test sample thought to contain the  
15 target nucleic acid sequence. These complementary nucleic acid sequences  
hybridize to one another under suitable conditions to form probe-target hybridization  
complexes which can be identified through the presence of the detectable label.  
Various methods in which particular aspects of this basic process have been  
optimized for the purpose of addressing specific assay requirements have developed  
20 over time. (see Wetmur, 1991, *Critical Reviews in Biochemistry and Molecular  
Biology* 26, 227-259).

While some homogeneous phase hybridization assays permit detection of the  
hybridized probe-target complex without removal of excess unhybridized probe  
sequences present in the assay solution (see Tyagi and Kramer, 1996, *Nature*  
25 *Biotechnology* 14, 303-308; Arnold, Waldrop, and Hammond, 1990, United States  
Patent No. 4,950,613), very sensitive assays require separation of the hybridized  
complex from the unhybridized probe prior to the detection step. Many different  
methods have been employed to accomplish this separation, including several that

rely on differences in physical characteristics between the two products. Other methods employ the use of a second nucleic acid sequence, described as a "capture" probe, for the purpose of separating the probe-target complex from the unhybridized probe. Capture probes are generally immobilized on a solid support and are selected  
5 to hybridize to a different portion of the target nucleic acid sequence than does the labeled probe. Thus, a tripartite capture probe-target-labeled probe complex forms which is bound to the solid support, while the unhybridized labeled probe remains unbound in solution, allowing the two products to be readily separated. Such assays are referred to as "sandwich" hybridizations (see, for example, Engelhardt and  
10 Rabbani, 1994, United States Patent No. 5,288,609). Although widely used, these assays require a number of steps to perform and are quite time-consuming.

Alternative assay methods, termed displacement assays, were developed in an attempt to simplify the method of identifying nucleic acids. A schematic illustration of a standard displacement assay is depicted in Figure 1 (see Diamond,  
15 S.E., *et al.*, 1988, United States Patent No. 4,766,062; Williams, J.I., *et al.* 1988, United States Patent No. 4,766,064; Vary, 1987, *Nucleic Acids Res.* 15, 6883-6897; Vary *et al.*, 1986, *Clinical Chemistry* 32, 1696-1701). In a standard displacement assay, a tether nucleic acid sequence, complementary to the target nucleic acid sequence, is hybridized to a shorter, detectably-labeled signal nucleic acid sequence,  
20 complementary to a specific subsequence of the tether sequence. The signal nucleic acid is fully base-paired with the tether nucleic acid in this signal-tether complex, but the longer tether nucleic acid retains a single-stranded region. Upon the introduction of a test sample containing the target nucleic acid, the target hybridizes to the single-stranded portion of the tether component. Since the target is  
25 homologous to the entire length of the tether, a homologous strand exchange reaction with the signal nucleic acid is initiated, and the target displaces the signal from the tether. This strand exchange reaction proceeds rapidly in the direction of signal nucleic acid displacement because the target is longer and forms a more stable duplex with the tether (see Green, C. and Tibbetts, C., 1981, *Nucleic Acids Res.* 9, 1905-1918). The amount of displaced labeled signal nucleic acid is measured to  
30 determine the amount of target nucleic acid in the sample. The tether component of

the probe complex can also be linked to a solid support, so that separation of the solid and solution phases results in isolation of the signal nucleic acid.

Unfortunately, despite their advantages, standard displacement assays do have certain drawbacks. For example, when target nucleic acid hybridizes to a  
5 tether not hybridized to a signal nucleic acid, or when a displaced signal nucleic acid hybridizes to a tether nucleic acid which was not previously hybridized to a signal nucleic acid, a decrease in the detection signal produced per unit of target hybridized results. Moreover, if the hybridized complex is not stable, an undesirable background signal can be introduced, which complicates interpretation of the assay  
10 results and reduces the sensitivity of the assay.

#### SUMMARY OF THE INVENTION

The present invention is based, at least in part, on the discovery of a probe-tether complex useful in a novel reverse displacement assay. This assay eliminates  
15 many of the difficulties inherent in the standard displacement assay, while retaining the key advantages over other solution phase hybridization reactions. The probe-tether complex of the invention is formed by contacting a first probe nucleic acid sequence complementary to the target nucleic acid sequence with a second tether nucleic acid sequence complementary to at least one subsequence of the first nucleic  
20 acid sequence under conditions suitable for hybridization between the two sequences. The probe-tether complex of the invention contains at least one double stranded segment and at least one single stranded segment.

In one aspect, the present invention pertains to a method for detecting the presence of a target nucleic acid in a test sample in which the sample to be tested for  
25 the presence of the target nucleic acid is introduced into a solution containing the probe-tether complex, under conditions suitable for hybridization to occur between the first probe nucleic acid sequence and the target nucleic acid sequence, so that the second tether nucleic acid sequence is displaced from the probe-tether complex, and a second complex is formed by the hybridization of the first probe nucleic acid  
30 sequence and the target nucleic acid sequence. The presence of the target nucleic acid can then be detected in the test sample.

In a preferred embodiment, one of the nucleic acid sequences contains a detectable label. In a particularly preferred embodiment, the probe nucleic acid contains a detectable label.

5 In a preferred embodiment, one of the nucleic acids is immobilized on a solid support. In a particularly preferred embodiment, the tether nucleic acid sequence is immobilized on a solid support.

In a preferred embodiment, one of the nucleic acids is immobilized on an electrophoretic medium. In a particularly preferred embodiment, the tether nucleic acid sequence is immobilized on an electrophoretic medium.

10 In another aspect, the present invention pertains to a method of detecting a target nucleic acid sequence in a test sample in which the method utilizes multiple sequential nucleic acid displacement reactions. In this method, the sample to be tested for the presence of the target nucleic acid is introduced into a solution containing the probe-tether complex, under conditions suitable for hybridization to  
15 occur between the first probe nucleic acid sequence and the target nucleic acid sequence, so that the tether nucleic acid sequence is displaced from the probe-tether complex, and a second hybridization complex, termed a displacement complex, is formed by the hybridization of the probe nucleic acid sequence and the target nucleic acid sequence. The tether nucleic acid sequence displaced by the target  
20 nucleic acid sequence is then available to form a probe-tether complex and the remainder of the steps of the method can be repeated. The sequence of steps can be repeated as many times as desired. The presence of the target nucleic acid can then be detected in the test sample.

In a preferred embodiment, one of the nucleic acid sequences contains a  
25 detectable label. In a particularly preferred embodiment, the probe nucleic acid contains a detectable label.

In a preferred embodiment, one of the nucleic acids is immobilized on a solid support. In a particularly preferred embodiment, the tether nucleic acid sequence is immobilized on a solid support.

30 In a preferred embodiment, one of the nucleic acids is immobilized on an electrophoretic medium. In a particularly preferred embodiment, the tether nucleic acid sequence is immobilized on an electrophoretic medium.

In yet another aspect, the present invention pertains to a kit for detecting the presence of a target nucleic acid sequence in a test sample. The kit contains a first nucleic acid sequence component complementary to the target nucleic acid and a second nucleic acid sequence component complementary to at least one subsequence  
5 of the first probe nucleic acid component, selected such that when the components are combined under conditions suitable for hybridization, the first and second components form a probe-tether complex containing at least one double stranded segment and at least one single-stranded segment. In a preferred embodiment, one of the nucleic acids can be immobilized on a solid support.

## 10 BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 is a schematic depiction of a standard displacement assay.

FIG. 2 is a schematic depiction of the reverse displacement assay of the present invention.

FIG. 3 is a depiction of a fluorimager scan of the results of the reverse  
15 displacement assay in a polyacrylamide gel as described in the Exemplification.

## DETAILED DESCRIPTION OF THE INVENTION

The invention described herein pertains to compositions and methods useful for analyzing nucleic acids in a variety of test samples. The methods of the invention described provide for a highly specific hybridization-based identification  
20 system for nucleic acids. The methods disclosed are based on the physical chemistry of hybridization between nucleic acids or polynucleotide sequence containing molecules. In particular, the methods utilize a reverse displacement type assay. The language "reverse displacement" is intended to include a reaction in which at least one product of the reaction is a complex formed by the hybridization  
25 of a probe nucleic acid and a target nucleic acid, and at least one product is formed by being displaced from a previous hybridization complex.

The language "test sample" is intended to include any sample that contains a protein, a nucleic acid or any charged species of molecule. For example, samples of biological origin such as blood, urine, other bodily fluids, cells (both plant and  
30 animal), cell extract, tissues and tissue extract are within the scope of the present

invention. Samples from non-biological sources are also within the scope of the present invention.

The language "nucleic acid" or "polynucleotide sequence" and their respective plurals are used essentially interchangeably herein and are intended to include deoxyribonucleic acid (hereinafter "DNA") and ribonucleic acid (hereinafter "RNA"). Both single stranded and double stranded nucleic acids are embraced by this invention. Higher ordered structures of nucleic acids, for example, RNA that has folded upon its linear strand forming a secondary loop structure, are also within the scope of the present invention. Nucleic acid sequences encompassed by the present invention can be from about 3 to about 10,000 nucleotides in length. There is no absolute minimum or maximum length requirement for target nucleic acid sequences, however, a preferred range is from about 10 to about 2,000, preferably about 30 to about 1,000, most preferably about 30 to about 100. Preferably, the probe and tether nucleic acid sequences are from about 10 to about 2,000 nucleotides in length, and about 5 to about 2,000 nucleotides in length, respectively, most preferably about 20 to about 100 and 6 to about 100. It should also be understood that the nucleic acid sequences of the present invention may be embedded within longer strands of nucleic acids or associated with other molecules. The directionality of the nucleic acids of the current invention may be either 5' to 3' or the reverse, that is, 3' to 5'.

The language "base-pairing" is intended to include those reactions which occur in an antiparallel manner as well as those which occur in a parallel fashion. Base-pairing itself is understood to essentially follow a complementary pattern wherein a purine pairs with a pyrimidine via hydrogen bonds. More particularly, it is understood that complementary base-pairing of individual base pairs generally follows Chargaff's Rule wherein an adenine pairs with a thymine (or uracil) and guanine pairs with cytosine. However, there are modified bases which account for unconventional base-pairing and these are considered to be within the scope of the instant invention.

The language "modified nucleic acid" is intended to include a DNA or RNA nucleic acid molecule that contains chemically modified nucleotides. The term "nucleic acid analogue" is intended to include non-nucleic acid molecules that can



engage in base-pairing interactions with conventional nucleic acids. These modified bases and nucleic acid analogues are considered to be within the scope of the instant invention. For example, nucleotides containing deazaguaine and uracil bases can be used in place of guanine and thymine, respectively, to decrease the thermal stability of hybridized probes. Similarly, 5-methylcytosine can be substituted for cytosine in hybrids if increased thermal stability is desired. Modification to the sugar moiety can also occur and is embraced by the present invention. For example, modification to the ribose sugar moiety through the addition of 2'-O-methyl groups can be used to reduce the nuclease susceptibility of RNA molecules. Modifications occurring with different moieties of the nucleic acid backbone are also within the scope of this invention. For example, the use of methyl phosphate, methyl phosphonate or phosphorothioate linkages to remove negative charges from the phosphodiester backbone can be used.

The best known example of a nucleic acid analogue is peptide nucleic acid (PNA), in which standard DNA bases are attached to a modified peptide backbone comprised of repeating N-(2-aminoethyl)glycine units (Nielsen *et al.*, *Science* vol. 254, pp. 1497-1500, 1991). The peptide backbone is capable of holding the bases at the proper distance to base pair with standard DNA and RNA single strands. PNA-DNA hybrid duplexes are much stronger than equivalent DNA-DNA duplexes, probably due to the fact that there are no negatively charged phosphodiester linkages in the PNA strand. In addition, because of their unusual structure PNAs are very resistant to nuclease degradation. The tight binding of PNA to natural nucleic acids suggests that it may be particularly useful in displacement assays. It will be apparent to those skilled in the art that similar design strategies can be used to construct other nucleic acid analogues that will have useful properties for displacement probe assays.

The language "hybridize" or "hybridization" is intended to include admixing of at least two nucleic acid sequences under conditions such that when at least two complementary nucleic acid sequences are present, they will form a double-stranded structure through base-pairing. The language "complement" or "complementary" is intended to describe nucleic acid sequences which can form a double-stranded structure through base-pairing. Mismatches are permitted in the instant invention.

Nucleotide mismatch can affect the affinity between nucleic acid sequences. The greater the mismatch between nucleic acid sequences, generally the lower the affinity between them as compared to perfectly matched nucleic acid sequences. Generally, the greater the mismatch between nucleic acid sequences, the more readily hybridization that exists between them can be disrupted. When mismatches between base pairs are present, they generally account for no more than 5% of the region of base-pairing. Preferably, the degree of complementarity between hybridization partners is from about 100% to about 95%, most preferably about 100%.

10        Altering the temperature at which the hybridization reaction occurs is a common method for modulating hybridization complex binding affinity. For example, at temperatures above the melting temperature ( $T_m$ ) of the hybridization complex, binding affinity will be low. Similarly, at temperatures below the  $T_m$ , binding affinity will be substantially higher. Therefore, if the  $T_m$  of a hybridization  
15        complex containing mismatches is determined and compared with the  $T_m$ s of complexes with shorter lengths of perfectly matched nucleotides, the effective pairing length of complexes with mismatches can be ascertained and applied to the present invention.

      The methods described herein comprise sequential steps involving the  
20        formation of hybridization complexes and displacement of nucleic acid sequences. Reference is now made to the Figures. Figure 1 depicts a standard displacement assay in which a target nucleic acid sequence (1) reacts with a tether-signal complex (2) to form a tether-target complex (3) product and a displaced signal (4) product. Figure 2 depicts a reverse displacement assay of the invention in which a target  
25        nucleic acid sequence (1) reacts with a probe-tether complex (5) to form a probe-target complex (6) product and a displaced tether (7) product. Figure 3 depicts a fluorimager scan of the results of the reverse displacement assay of the present invention in a polyacrylamide gel as described in the Exemplification. The probe nucleic acid sequence (8) is represented at the top of the figure with the target  
30        nucleic acid sequence (1) directly underneath. The probe-target complex (6) appears as a discrete band below the layer of immobilized probe-tether complex (5).

In the reverse displacement reaction of the invention, initially, a probe-tether nucleic acid complex is formed when a probe nucleic acid sequence and a tether nucleic acid sequence are combined, under conditions suitable for hybridization, such that the complex contains at least one double stranded segment and at least one  
5 single stranded segment. The first nucleic acid sequence is the probe nucleic acid sequence. The language "probe" is intended to include a nucleic acid sequence complementary to the target nucleic acid sequence. The second nucleic acid sequence is the tether nucleic acid sequence. The language "tether" is intended to include a nucleic acid sequence complementary to at least one subsequence of the  
10 probe nucleic acid. The language "subsequence" is intended to include any contiguous segment of a larger sequence. Thus, the tether nucleic acid is identical, or at least substantially similar in sequence, to a specific subsequence of the target nucleic acid. The tether nucleic acid can contain more than one subsequence complementary to the probe nucleic acid. In addition, a complementary  
15 subsequence can be of any length. However, the entire length of the tether nucleic acid should not be complementary to the entire length of the probe nucleic acid.

When this probe-tether nucleic acid complex is combined in solution with the target nucleic acid under suitable conditions, the single stranded region of the probe-tether nucleic acid hybridizes with the target nucleic acid, and the probe  
20 nucleic acid is displaced from the tether nucleic acid by homologous strand exchange. One product of the reverse displacement reaction is a hybrid of the probe nucleic acid and the target nucleic acid. The language "probe-target complex" is intended to include the hybridized nucleic acid product of this reverse displacement reaction. A second product of the reverse displacement reaction is a displaced tether  
25 nucleic acid. A schematic illustration of a reverse displacement reaction of the invention is shown in Figure 2.

As can be readily understood, two types of hybridization complexes are formed in the methods of the present invention. The first is a probe-tether hybridization complex and the second is a probe-target hybridization complex. The  
30 second complex can also be described as a displacement complex.

The probe-tether complex of the invention can be formed in an aqueous medium under conditions suitable for nucleic acid hybridization. Nucleic acid

sequences are selected for this assay based upon their ability to hybridize to the other nucleic acid sequences utilized in the assay. The assay does not depend on a particular length requirement for any nucleic acid. Preferably, however, the binding region between the nucleic acid sequences undergoing hybridization to form a probe-tether complex is from about 5 to about 2,000 nucleotides in length, most preferably about 10 to 200 nucleotides in length. Additionally, for the probe-tether complex, the first probe nucleic acid sequence will have a degree of complementarity 95 to 100% with the target nucleic acid sequence, while the second tether nucleic acid sequence will have a degree of complementarity 95 to 100% to a subsequence of the first probe nucleic acid sequence, to the extent that such is required to facilitate the requisite chemical hybridization reaction.

A desirable temperature range during the formation of the probe-tether complex of the invention is from about 15°C to about 90°C, depending upon the variables of other properties of the solution, such as ionic strength, pH, nucleic acid sequence concentration, degree of complementarity of the sequences, as well as other components of the aqueous medium that may affect melting temperatures. For a discussion and examples of suitable conditions see Wetmur, 1991, *Critical Reviews in Biochemistry and Molecular Biology*, 26:227-259.

The language "displacement complex", which is used interchangeably with the language "probe-target complex", is intended to include one product of a reaction event in which at least one nucleic acid sequence is displaced or removed from a hybridization complex. Such a complex is formed by the hybridization of two or more nucleic acid sequences. There is no particular length requirement for participating nucleic acid sequences; however, the length of the nucleic acid sequences do affect the stability of the hybridization complex. The size required to achieve a desired level of stability can be determined empirically. Preferably, the binding region between the nucleic acid sequences undergoing hybridization to form a displacement complex is from about 5 nucleotides to about 2,000 nucleotides in length, most preferably about 15 to 200. In the reverse displacement method of the present invention, the target nucleic acid sequence is one component of the displacement complex. The complex is formed as a result of the previous probe-tether complex coming into contact with the competing target nucleic acid sequence.

Because the competing target nucleic acid sequence has a higher affinity for the probe nucleic acid sequence, the tether nucleic acid sequence is displaced from the complex. The product of this event is not only a new hybridization complex, but also, the displaced tether nucleic acid sequence.

5           The reaction events associated with a displacement complex can occur in an aqueous medium in which conditions are suitable for hybridization. The appropriate temperature to perform displacement reactions depends on the thermal stabilities of the hybrids present in the hybridization complex.

          Either of the components of the probe-tether complex can be immobilized on  
10 a solid support through the use of a ligand. Preferably, the tether component is immobilized, since doing so maintains the advantages provided by the most efficient standard displacement assays. When the tether is immobilized, hybridization of the probe nucleic acid to the target nucleic acid will result in a release of the probe-target complex into solution. Thus, analysis of the isolated probe-target complex  
15 can be readily performed subsequent to separation of the solid and liquid phases. If the tether contains an affinity tag, separation of displaced probe from nondisplaced probe can be achieved following capture of the affinity tag on solid support material containing binding ligands specific for the tag.

          The language "ligand" is intended to include any molecule that can form a  
20 specific binding complex with a target nucleic acid and that can be immobilized upon a suitable solid support.

          One especially useful example of a ligand is a single stranded nucleic acid, which can bind, for example, to a complementary nucleic acid by hybridization. Single stranded nucleic acids can also be used for isolation of duplex nucleic acids  
25 by triplex formation (Hogan and Kessler, United States Patent No. 5,176,966; Cantor, *et al.*, United States Patent No. 5,482,836). Double stranded nucleic acids can also serve as useful ligands for nucleic acid binding proteins, or for target nucleic acid sequences that bind to the ligand by triplex or tetraplex formation.

          Nucleic acid aptamers (Tuerk and Gold, *Science* 1990, 249, 5050-510.;  
30 Joyce, *Gene* 1989, 82, 83-87.; Ellington and Szostak, *Nature* 1990, 346, 818-822) can also be used as affinity ligands in the methods of the present invention. The language "aptamer" is intended to include a nucleic acid ligand that is selected for

its ability to fit a target protein or other polymer. Aptamers can be selected against many kinds of targets, including proteins, small organic molecules, and carbohydrates (see Klug and Famulok, *Molecular Biology Reports* 1994, 20, 97-107). Thus, selection of aptamer ligands offers a simple and flexible mechanism  
5 for obtaining affinity ligands against virtually any target molecule.

Other useful ligands include proteins which can bind to specific targets. An especially useful class of protein ligands are antibody molecules, which can be elicited against a wide range of targets by immunization methods. Similarly, receptor proteins may be useful as ligands for purification and detection of targets  
10 that bind to or are bound by them.

Carbohydrates have been successfully used as affinity ligands for electrophoretic purification of lectins (Horejsi and Kocourek, *Biochim. Biophys. Acta* 1974, 336, 338-343), and may be useful for purification and detection of molecules that bind to specific carbohydrates or glycoproteins.

15 Many other types of immobilized ligands are possible including peptides, amino acids, nucleosides, nucleotides, small organic molecules, lipids, hormones, drugs, enzyme substrates, enzyme inhibitors, enzymes, coenzymes, inorganic molecules, chelating agents, macromolecular complexes, polysaccharides, monosaccharides, and others.

20 Immobilization of the nucleic acid can be accomplished by direct or indirect attachment to a solid support. The solid support can be a chromatographic support, a thin-layer plate or membrane, or electrophoretic polymeric media.

Any medium suitable for electrophoresis can be used in the methods of the present invention. In general, suitable media fall into two classes. The first includes  
25 media composed of gel-forming materials like crosslinked polyacrylamide and agarose. The second class includes media composed of solutions of linear noncrosslinked polymers such as polyacrylamide, poly(hydroxyethylcellulose), and poly(ethyleneoxide). The latter category is commonly used for capillary electrophoresis applications.

30 Direct attachment to the polymeric components of the medium can be accomplished by the formation of covalent bonds between the ligand and the polymer. Noncovalent binding between the ligand and polymer substituents can also

be used. For instance, strong noncovalent binding provided by the widely-used biotin-streptavidin and digoxigenin-antidigoxigenin systems can be used to attach ligands to appropriately modified polymeric media. Covalent attachment is generally preferred.

- 5           Direct connection between the polymeric medium and the ligand are not strictly required. For instance, ligands can be attached to particulate supports, such as microspheres, and the particulate supports can be immobilized within the polymer medium by physical entrapment (Cantor, *et al.*, United States Patent No. 5,482,863). The particles may be macroscopic, microscopic, or colloidal in nature, (see
- 10 Polysciences, Inc., 1995-1996 Particle Catalog, Warrington, PA).

In a similar manner, ligands can be attached to highly branched soluble polymers. Due to their branched shape, such ligand-polymer complexes display extremely large effective hydrodynamic radii and, therefore, will not migrate upon electrophoresis in many kinds of polymeric media of appropriately small pore size.

- 15 Thus, they can be entrapped within the media in the same fashion as particulate supports.

- Absolute immobilization of the ligand within the medium is not required for all embodiments of the invention. For many applications, it is sufficient that the mobility of the target is changed upon formation of a binding complex with the
- 20 ligand. This condition can be satisfied by coupling the ligand to a medium component that has extremely low electrophoretic mobility. However, for efficient purification the change in mobility should be as large as possible. Therefore, media utilizing true immobilization of the ligand within the medium are generally preferred for use in this invention.

- 25 Commonly used gel media useful for the present invention include acrylamide and agarose gels. However, other materials may be used. Examples include modified acrylamides and acrylate esters (for examples see Polysciences, Inc., Polymer & Monomer catalog, 1996-1997, Warrington, PA), starch (Smithies, *Biochem. J.* 1959, 71, 585; product number S5651, Sigma Chemical Co., St. Louis,
- 30 MO), dextrans (for examples see Polysciences, Inc., Polymer & Monomer Catalog, 1996-1997, Warrington, PA), and cellulose-based polymers (for examples see Quesada, *Current Opinions in Biotechnology* 1997, 8, 82-93). An acrylamide gel

matrix such as Acrydite<sup>TM</sup>-phosphoramidite media (Mosiatic Technologies, Boston, MA) is a particularly preferred matrix. Any of these polymers can be chemically modified to allow specific attachment of ligands (including nucleic acids, proteins, peptides, organic small molecules, and others) for use in the present invention.

5 For some methods, it may be useful to use composite media, containing a mixture of two or more supporting materials. An example is the composite acrylamide-agarose gel. These gels typically contain from 2-5% acrylamide and 0.5%-1% agarose. In these gels the acrylamide provides the chief sieving function, but without the agarose, such low concentration acrylamide gels lack mechanical  
10 strength for convenient handling. The agarose provides mechanical support without significantly altering the sieving properties of the acrylamide. In such cases, the nucleic acid can be attached to the component that confers the sieving function of the gel, since that component makes the most intimate contact with the solution phase target nucleic acid.

15 For capillary electrophoresis (CE) applications it is convenient to use media containing soluble polymers. Examples of soluble polymers that have proven to be useful for CE analyses are linear polymers of polyacrylamide, poly(N,N-dimethylacrylamide), poly(hydroxyethylcellulose), poly(ethyleneoxide) and poly(vinylalcohol) as described in Quesada, *Current Opinion in Biotechnology*  
20 1997, 8, 82-93. Solutions of these polymers can also be used to practice the methods of the present invention.

Methods of coupling a variety of ligands to create affinity electrophoresis media are well known to those skilled in the art. Many ligands can be coupled to agarose, dextrans, cellulose, and starch polymers using cyanogen bromide or  
25 cyanuric chloride activation. Polymers containing carboxyl groups can be coupled to ligands that have primary amine groups using carbodiimide coupling. Polymers carrying primary amines can be coupled to amine containing ligands with glutaraldehyde or cyanuric chloride. Many polymers can be modified with thiol-reactive groups which can be coupled to thiol-containing ligands. Many other  
30 suitable methods are known in the literature. For examples, see Wong, "Chemistry of Protein Conjugation and Cross-linking", CRC Press, Boca Raton, FL, 1993.



Methods for covalently attaching ligands by copolymerization with the polymeric material of the electrophoretic medium have also been developed. In this approach, ligands are chemically modified with a copolymerizable group. When such modified ligands are copolymerized with suitable mixtures of polymerizable monomers, polymeric media containing high concentrations of immobilized ligand can be produced. Examples of methods for covalently attaching nucleic acids to polymerizable chemical groups are found in United States Patent Application Serial No. 08/812,105, filed March 7, 1997, entitled "Nucleic Acid-Containing Polymerizable Complex," the teachings of which are expressly incorporated by reference herein, in their entirety. Similar methods have been used to immobilize proteins and small organic molecules within polymer layers and gels (Bille *et al.*, *Eur. J. Biochem.* 1989, 180, 41-47; Wang *et al.*, *Nature Biotechnology* 1997, 15, 789-793; Holtz and Asher, *Nature* 1997, 389, 829-832).

Other approaches for attaching nucleic acid probes to preformed polyacrylamide polymers, including gels or linear soluble polymers can be found in Timofeev *et al.*, *Nucleic Acids Res.* 1996, 24, 3142-3148 and Ghosh and Fahy, United States Patent No. 5,478,893.

One important advantage provided by the reverse displacement method of the present invention is that saturation of the tether nucleic acid with labeled probe nucleic acid is not required. Because the tether nucleic acid is not complementary to the target nucleic acid, uncomplexed tether nucleic acid will not hybridize with the target and, therefore, does not compete with target nucleic acid for hybridization with probe nucleic acids. In point of fact, since uncomplexed tether nucleic acids can hybridize to probe nucleic acids that have been released in a target-independent manner, it is likely that background noise is reduced in this assay. Furthermore, unhybridized tether nucleic acids cannot hybridize to probe-target hybrids, a significant difference from the corresponding situation in the standard displacement reaction.

In a preferred embodiment of the invention, the probe nucleic acid and/or the tether nucleic acid contains a detectable label. Most preferably, the probe nucleic acid contains a detectable label. The label can be bound ionically, covalently or in any other appropriate manner known in the art. The label is preferably bound to any

region of the nucleic acid comprising the probe or tether nucleic acid sequences.

The language "label" is intended to include those which directly utilize radioactive isotopes, fluorescent moieties, chemiluminescent moieties, and direct enzyme conjugates, as well as indirect labels, such as affinity labels for use with secondary or tertiary labeled molecules. Because the reverse displacement assay is not label-specific, any practicable label known to those of skill in the art is suitable for use with the assay.

Detection of the target nucleic acid may be accomplished in a variety of ways, but generally through detecting and/or measuring the value or change in value of any chemical or physical property of the product of the reverse displacement assay. Examples of physical properties suitable for use in detection and/or measurement include mass or density properties as determined by mass spectrometry or plasmon resonance, optical properties as determined by methods detecting emission, absorption, fluorescence, phosphorescence, luminescence, chemiluminescence, polarization, and refractive index changes, or other known techniques, electrical properties as determined by methods detecting conductivity, absorption or emission of other electromagnetic energy, radioactive properties, as well as induced changes in solution properties such as viscosity, turbidity, and optical rotation.

The reverse displacement assay as described above and illustrated in Figure 2 includes a single displacement reaction. However, multiple sequential reactions are also within the scope of the present invention. In a reaction of this type, for example, a probe nucleic acid may be affinity labeled with a single-stranded nucleic acid tail. The probe-target complex may then be transferred to a secondary reaction area where this tail serves as the target for a second reverse displacement reaction.

Moreover, several cycles of probe and displacement complex formation, generating multiple displacement nucleic acid sequences in which one of the displaced nucleic acid sequences is identical to an original nucleic acid sequence, thereby facilitating cycling back to the first displacement complex and proceeding through the sequential hybridization and displacement cycles again can be performed. This process of recycling through probe and displacement complex formation provides for the amplification of assay signal prior to detection. The use

of such multiple sequential displacement reactions has been previously described in co-pending United States Patent Application with Serial No. 09/188,086 entitled "Multiple Sequential Polynucleotide Displacement Reactions for Signal Amplification and Processing," filed on November 6, 1998, the teachings of which  
5 are expressly incorporated by reference herein in their entirety.

The present invention further pertains to a kit used for determining the presence of a target nucleic acid sequence within a test sample. The kit contains a first probe nucleic acid sequence component complementary to the target molecule and a second tether nucleic acid sequence component complementary to at least one  
10 subsequence of the probe component. The first and second components are selected such that when admixed, under conditions suitable for hybridization, the first and second components form a probe-tether complex containing at least one double stranded segment and at least one single stranded segment. The components may be provided singly or in combination. The kit may also include additional components.  
15 In a preferred embodiment, the probe and/or the tether component are detectably labeled. In a most preferred embodiment, the probe component is detectably labeled.

The invention is further illustrated by the following non-limiting example. The contents of all the patents, patent applications and other references cited herein  
20 are expressly incorporated by reference in their entireties.

#### EXEMPLIFICATION

The reverse displacement assay of the invention was demonstrated as follows. A naturally occurring sequence from the 16S rRNA subunit of *E. coli* was selected as the target nucleic acid. A synthetic DNA oligonucleotide was used as a  
25 model target, 5'-CACAAGCGGTGG-AGCATGTGGTTTAATTCGATGCAACGCGAAGAACC'TTACC. The unlabeled oligonucleotide tether 5'-Ac-CACAAGCGGTGGAGCATGTGGTTT was synthesized, where Ac represents an Acrydite<sup>TM</sup> phosphoramidite. The Acrydite<sup>TM</sup> group allows immobilization in an acrylamide gel matrix by co-polymerization. A  
30 fluorescently labeled probe oligonucleotide was synthesized with the sequence 5'-cy3-GGTTCTTCGCGTTGCATCGAATTAAACCACATGCTCCACCGCTTGT

GCAAAAACGATAAACCAACCA, of which the first forty-seven (47) nucleotides are complementary to the target. The last twenty (20) nucleotides were added as an affinity tag to allow the probe-target complex to be captured after the displacement reaction, although the tag was not utilized in this experiment.

5           The unlabeled tether oligonucleotide was copolymerized at a concentration of 10.0  $\mu$ M in a polyacrylamide slab gel (10.0 cm x 10.0 cm x 0.8 mm) as a band approximately 1.0 cm wide extending across the width of the gel. The labeled oligonucleotide probe was then bound to this immobilized tether by electrophoresing either 1.0 pmol or 5.0 pmol through the gel at 50 V for 30 minutes. Unbound probe  
10 oligonucleotide and non-immobilized tether were removed from the gel by electrophoresing in the reverse direction at 200 V for 1 hour. Samples were then prepared that contained 0.0, 5.0, 1.0, 0.2, 0.04 or 0.008 pmol of the synthetic target. These samples were loaded on the gel and electrophoresed in the forward direction at 50 V for 1 hour. The gel was subsequently scanned using a Molecular Dynamics  
15 Fluorimager and the results are shown in Figure 3. The complex of target with labeled probe appears as a discrete band below the layer of immobilized tether oligonucleotide with bound labeled probe. The amount of probe-target complex was found to be linearly dependent on the amount of target loaded in each lane of the gel.

          This experiment clearly demonstrates that a saturated layer is not needed for  
20 this new assay method. In addition, labeled oligonucleotide that dissociates from the immobilized oligonucleotide in a non-target dependent manner was rebound at a new site lower in the immobilized oligonucleotide layer. This decreases the background in the assay.

#### EQUIVALENTS

25           While this invention has been particularly shown and described with reference to preferred embodiments thereof, it will be understood by those skilled in the art that various changes in form and details may be made therein without departing from the spirit and scope of the invention as defined by the appended claims. Those skilled in the art will recognize or be able to ascertain using no more  
30 than routine experimentation, many equivalents to the specific embodiments of the invention described specifically herein. Such equivalents are intended to be encompassed by the scope of the claims.

## CLAIMS

What is claimed is:

1. A method of detecting the presence of a target nucleic acid sequence in a test sample comprising the following steps:
  - 5 a) forming a probe-tether complex by contacting a first probe nucleic acid sequence comprising a sequence complementary to the target nucleic acid sequence with a second tether nucleic acid sequence comprising a sequence complementary to at least one subsequence of the first probe nucleic acid sequence, under conditions suitable for  
10 hybridization between the first and the second nucleic acid sequences, thereby forming a probe-tether complex containing at least one double stranded segment and at least one single stranded segment;
  - b) introducing the test sample into a solution containing the probe-tether complex, under conditions suitable for hybridization between the first  
15 probe nucleic acid sequence and the target nucleic acid sequence, thereby displacing the second tether nucleic acid sequence from the probe-tether complex;
  - c) forming a probe-target complex by contacting the first probe nucleic acid sequence with the target nucleic acid sequence, under conditions  
20 suitable for hybridization between the first probe nucleic acid sequence and the target nucleic acid sequence, thereby forming a probe-target complex; and
  - d) detecting the presence of the target nucleic acid sequence in the test  
25 sample.
2. The method of Claim 1, wherein one of the nucleic acid sequences contains a detectable label.

-20-

3. The method of Claim 2, wherein the probe nucleic acid sequence contains a detectable label.
4. The method of Claim 3, wherein the mode of detecting the signal is selected from the group consisting of mass spectrometry, plasmon resonance, optical  
5 emission, absorption, fluorescence, phosphorescence, luminescence, chemiluminescence, polarization, refractive index changes, electrical conductivity, radioactivity, viscosity, turbidity and optical rotation.
5. The method of Claim 1, wherein the nucleic acid sequences forming the probe-tether complex are selected from the group consisting of nucleic acids,  
10 modified nucleic acids and nucleic acid analogs.
6. The method of Claim 1 further comprising a step of separating the displaced tether nucleic acid sequence from the probe-target complex.
7. The method of Claim 6, wherein the tether sequence is immobilized on a solid support.
- 15 8. The method of Claim 7, wherein the solid support is selected from the group consisting of chromatographic supports, thin-layer plates and membranes, and electrophoretic media.
9. The method of Claim 8, wherein the electrophoretic media is selected from the group consisting polyacrylamide gel, starch gel, agarose gel,  
20 polyacrylamide, poly(hydroxethylcellulose), and poly(ethyleneoxide).
10. A method of detecting a target nucleic acid sequence in a test sample comprising multiple sequential nucleic acid displacement comprising the following steps:
  - a) forming a probe-tether complex by contacting a first probe nucleic  
25 acid sequence comprising a sequence complementary to the target nucleic acid sequence with a second tether nucleic acid sequence

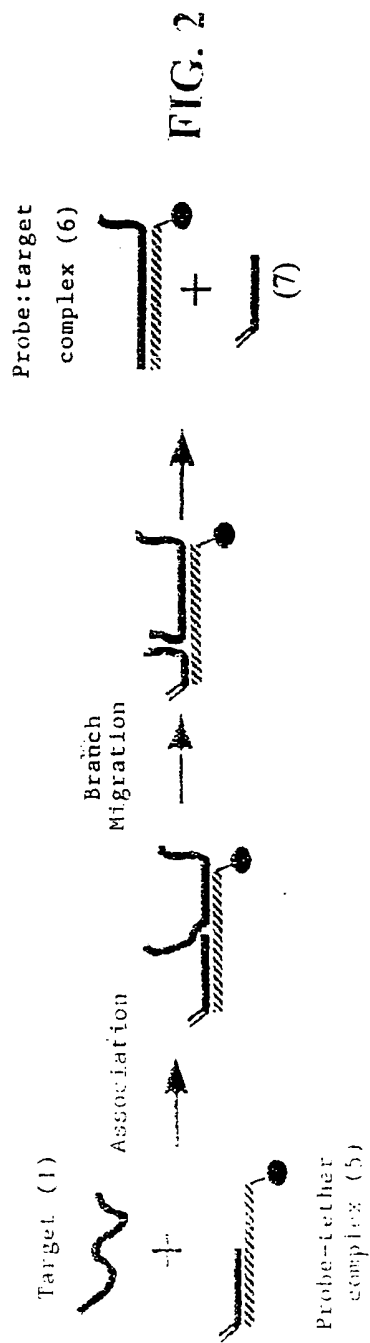
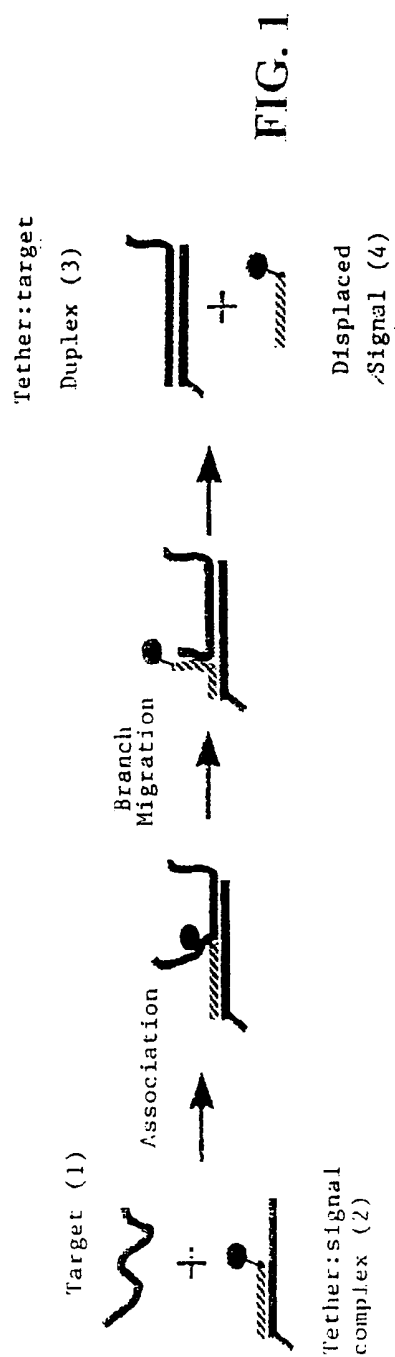
- comprising a sequence complementary to at least one subsequence of the first probe nucleic acid sequence, under conditions suitable for hybridization between the first and the second nucleic acid sequences, thereby forming a probe-tether complex containing at least one double stranded segment and at least one single stranded segment;
- 5
- b) immobilizing the probe-tether complex to a solid support;
- c) introducing the test sample into a solution in contact with the immobilized probe-tether complex, under conditions suitable for hybridization between the first probe nucleic acid sequence and the target nucleic acid sequence, thereby displacing the second tether nucleic acid sequence from the probe-tether complex;
- 10
- d) forming a probe-target complex by contacting the first probe nucleic acid sequence with the target nucleic acid sequence, under conditions suitable for hybridization between the first probe nucleic acid sequence and the target nucleic acid sequence, thereby forming a probe-target complex;
- 15
- e) repeating steps a) through d) with the displaced tether product formed in step (d); and
- 20
- a) detecting the presence of the target nucleic acid sequence in the test sample.
11. The method of Claim 10, wherein one of the nucleic acid sequences contains a detectable label.
12. The method of Claim 11, wherein the probe nucleic acid sequence contains a detectable label.
- 25
13. The method of Claim 11, wherein the mode of detecting the signal is selected from the group consisting of mass spectrometry, plasmon resonance, optical emission, absorption, fluorescence, phosphorescence, luminescence,

chemiluminescence, polarization, refractive index changes, electrical conductivity, radioactivity, viscosity, turbidity and optical rotation.

14. The method of Claim 10, wherein the nucleic acid sequences forming the probe-tether complex are selected from the group consisting of nucleic acids, modified nucleic acids and nucleic acid analogs.
15. The method of Claim 10 further comprising a step of separating the displaced tether nucleic acid sequence from the probe-target complex.
16. The method of Claim 10, wherein the solid support is selected from the group consisting of chromatographic supports, thin-layer plates and membranes, and electrophoretic media.
17. A kit for detecting the presence of a target nucleic acid sequence in a test sample comprising:
- a) a first probe nucleic acid sequence component complementary to the target molecule; and
  - b) a second tether nucleic acid sequence component complementary to at least one subsequence of the probe component;
- wherein, when combined under conditions suitable for hybridization, the first and second components form a probe-tether complex containing at least one double stranded segment and at least one single-stranded segment.
18. The kit of Claim 17 further comprising a component with a detectable label.
19. The kit of Claim 17, wherein the components of the probe-tether reagent complex are selected from the group consisting of nucleic acids, modified nucleic acids and nucleic acid analogs.
20. The kit of Claim 17, wherein one of the components can be attached to a solid support.



21. The kit of Claim 20, wherein the tether component can be attached to a solid support.



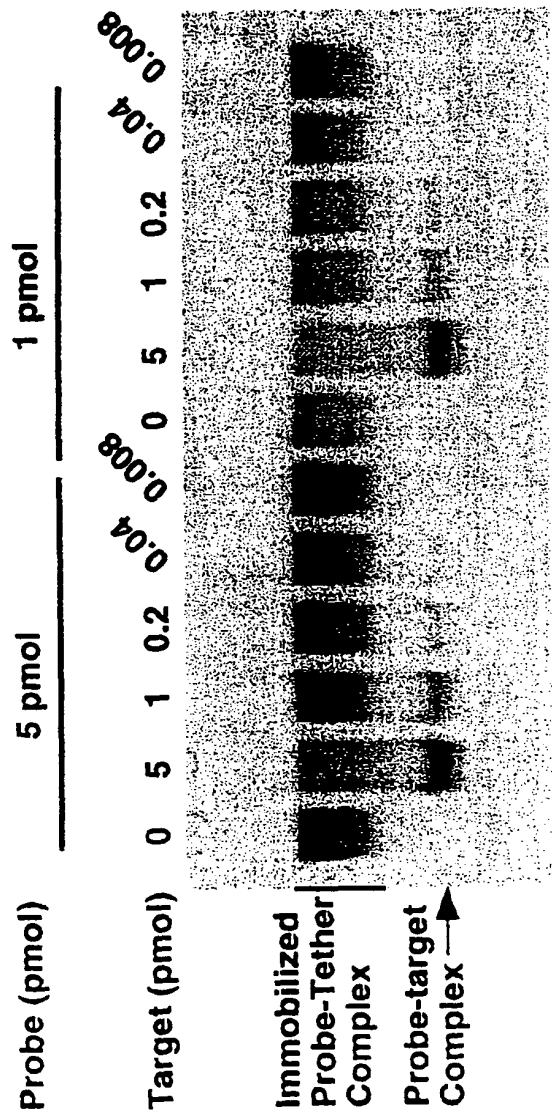


Figure 3.

# INTERNATIONAL SEARCH REPORT

International Application No.

PCT/US 99/23035

## A. CLASSIFICATION OF SUBJECT MATTER

IPC 7 C12Q1/68

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 7 C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 87 03911 A (GENETICS INST) 2 July 1987 (1987-07-02) figure 1	1-7, 17-21
X	VARY ET AL.: "Nonisotopic detection methods for strand displacement assay of nucleic acids" CLIN. CHEM., vol. 32, no. 9, 1986, pages 1696-1701, XP002131658 cited in the application the whole document	1-6, 17-21
	-/-	

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

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"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

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Date of the actual completion of the international search

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# INTERNATIONAL SEARCH REPORT

Int. Appl. No.

PCT/US 99/23035

## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
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